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Out of the Mainstream: Research Frontiers

In the previous chapters, I have discussed what may be thought of as mainstream applications of flow technology. Cells stained for surface and intracellular antigens and nuclei stained for DNA content together constitute a large majority of the particles that flow through the world's cytometers. As noted in the Preface, however, flow cytometry has continued to surprise everyone with its utility in unusual and unpredictable fields of endeavor. By the time this book appears in print, some new applications will almost certainly have progressed into the flow mainstream and other newer applications will have taken their place in the tributaries (is it time to kill this metaphor?).

At present, the large number of "tributary" applications (highly varied, often idiosyncratic, and sometimes unsung) precludes any attempt at making a chapter in an introductory book such as this into a complete catalogue. Therefore, I will attempt here only to hint at a few applications that seem to me to be important, or unusual, or to show promise (it has not escaped my attention that several of the applications that I said showed promise 10 years ago are still included in this chapter as holding potential ...). Because each reader will have his or her own personal goals for the use of flow cytometry according to his or her own interests, I think it is important here mainly to convey some feeling for the enormous variety and surprising potential that are found in laboratories using flow cytometry as a research tool.

FUNCTIONAL ASSAYS

Activation Markers

Functional analyses can be performed on the flow cytometer by assaying cells at different times during the progress of a stimulated or ongoing change. For example, although leukocytes express certain proteins on their surface that can be thought of as phenotypic markers (in other words, these markers are relatively constant in intensity and are expressed dependably by certain subgroups of cells), other proteins come and go on the cell surface, depending on the cell's functional/physiological/activation state. These surface proteins (the interleukin-2 receptor [CD25] or CD69 or CD154 on T cells are good examples for leukocytes) show characteristic time courses. Time of appearance after stimulation varies, and expression of some is long-lasting and of others is relatively transient (Fig. 11.1). In addi-

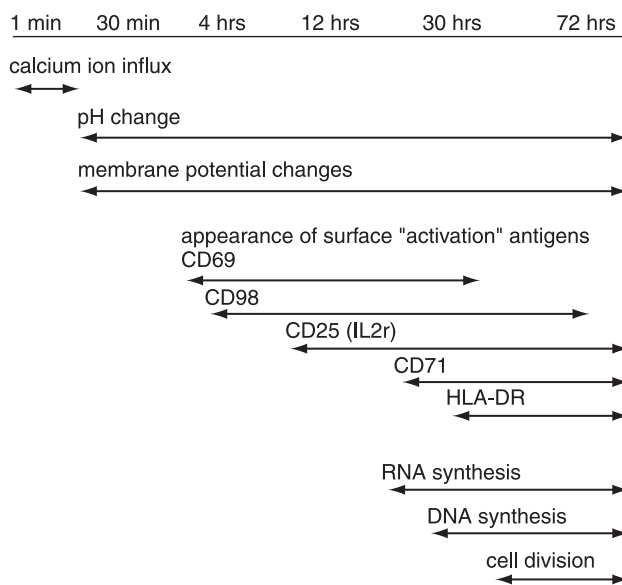


Fig. 11.1. Lymphocyte responses to stimulation, as measurable by flow cytometry. The sequence and duration of responses are approximate and will depend on the nature and strength of the trigger. Adapted from Shapiro (1995).

tion, there are internal proteins that are synthesized in response to cell activation: The cytokines discussed in the chapter on intracellular proteins are in this class, as are the cyclins discussed in the DNA chapter.

This pattern of progressive and/or transient expression raises certain problems for a flow cytometrist interested in knowing about the physiological state of a cell. Obviously, the time at which one assays the cells is critical, but it should also be clear that any protein that comes and goes will also, at certain times, be expressed at very low levels on the cell surface. Therefore it becomes essential to know something about the sensitivity of the flow cytometer. A cell may appear to be negative for a certain protein if it is assayed on a certain cytometer using a certain fluorochrome-conjugated antibody, but the protein might be detectable if a more sensitive cytometer or a brighter fluorochrome is used. The other issue with so-called activation markers is that the level of expression becomes the relevant read out (Fig. 11.2). We become interested not just in whether a cell is positive but in how positive it is (the median or mean intensity rather than the proportion of positive cells). Calibration of the fluorescence scale also becomes more important if we want to compare intensity values from day to day or from laboratory to laboratory.

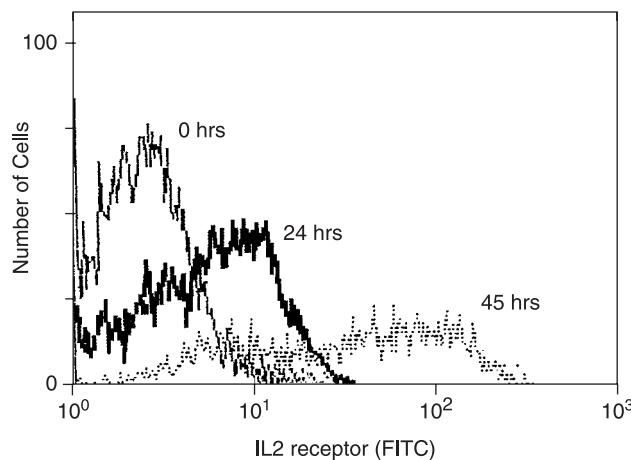


Fig. 11.2. The gradual increase in expression of interleukin-2 receptors on the surface of lymphocytes after phytohemagglutinin stimulation.

Precursor Frequencies

By way of a completely different type of functional analysis, we can look at the use of tracking dyes to label and follow cells. Cells can be stained with lipophilic fluorochromes or with fluorochromes that bind nonspecifically to all proteins. An example of the lipophilic fluorochromes are the so-called PKH dyes that insert themselves into the bilayer of cell membranes. The dye carboxyfluorescein succinimidyl ester (CFSE) binds to the free-amino groups on all cell proteins. These two types of dyes can be used to stain cells with considerable stability. The stained cells can then be injected into an animal and tracked to their homing location.

In a different use of this technique, the proliferation history of a cell can be followed. Because the dye is passed on from a parent to each of its daughter cells, a halving dilution of dye content occurs at each cell division (and a resulting halving of intensity is seen). In this way, according to their intensity, the cells in a suspension can be classified as to how many divisions they have undergone since their labeling at the beginning of the experiment. Those with half the original intensity have undergone one cell division; those with one eighth of the original intensity have undergone three cell divisions; and so forth. By analysis of the intensity histogram of a population of cells after several days incubation with a stimulus (knowing that a cell's intensity gives us the number of divisions undergone by that cell), back-calculation can tell us what proportion of cells in the original population were stimulated to divide (Fig. 11.3). This method is therefore a way to look at the precursor frequency of cells responding to various specific stimuli and a way to look at the past proliferative history of a given cell.

Kinetics

Whereas the applications discussed so far in this book have dealt with ways to describe cells or nuclei that have been stopped in their tracks for analysis at a given moment, flow cytometry has also been used to follow the physiological function of cells in true kinetic analysis. As an example of this kind of analysis, we can discuss the use of a flow

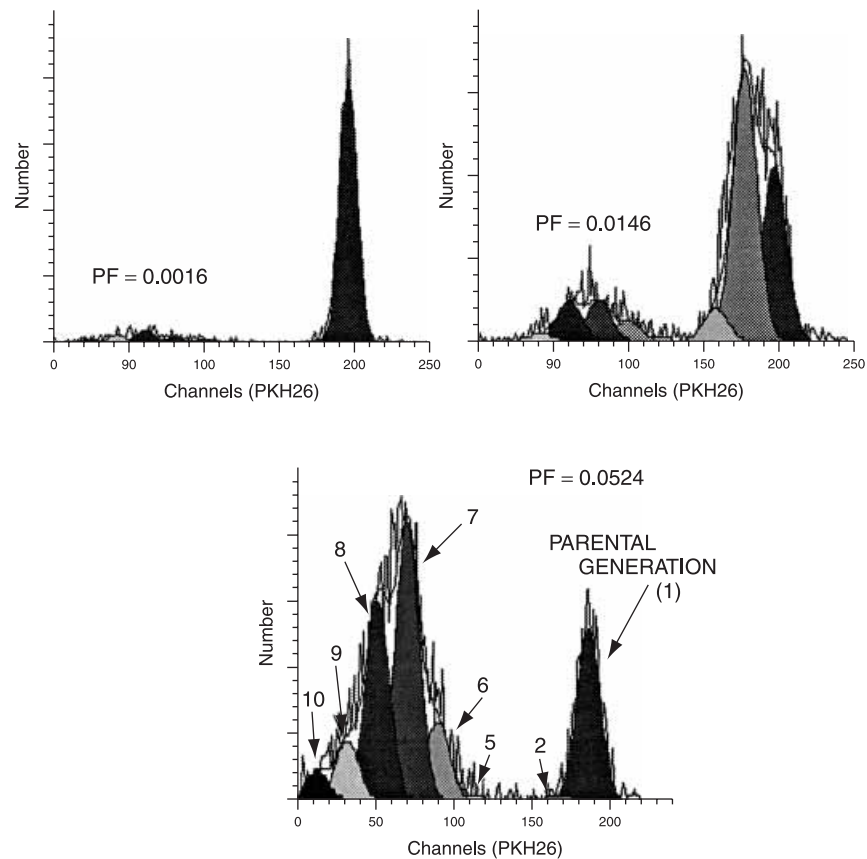


Fig. 11.3. The analysis of a histogram of PKH26 fluorescence to categorize cells according to their generation number (1–10) after 10 days culture. As cells divide, the PKH dye becomes diluted by half, with each halving of intensity marking cells as having gone through another division. The precursor frequencies (PF) of the dividing cells can be calculated. Three examples are shown with lymphocytes from donors with low, medium, and high responses to the tetanus toxoid antigen. Data from Jan Fisher and Mary Waugh.

cytometer to look at the rapid changes in calcium ion concentration that occur when cells become physiologically activated.

One of the early responses made by many types of cells to a stimulus is a rapid influx of calcium ions across the plasma membrane and a resulting increase in the cytoplasmic level of free ionic calcium.

This increase is thought to be one of the early steps involved in so-called signal transduction and can result in the activation of enzyme systems responsible for subsequent metabolic or developmental changes. Lymphocytes show increases in intracellular calcium in response to many kinds of specific and nonspecific surface ligand binding, some of which lead to the cellular changes that we associate with an immune response. Many other classes of cells also show calcium changes in response to stimulation.

A range of dyes developed by Roger Tsien in California has been useful in flow cytometry because they can be loaded into living cells where they will chelate calcium in a reversible equilibrium and fluoresce in proportion to their calcium load. A dye called *fluo-3* absorbs light from the 488 nm line of the argon laser and fluoresces little in the absence of calcium but significantly (at 530 nm) when binding the ion. However, the use of *fluo-3* is difficult to standardize because the amount of fluorescence varies with the amount of dye loaded into the cells. A more useful dye is the related indo-1, which, although it requires a light source with ultraviolet output for excitation (a high-power argon laser or a mercury arc lamp, for example), permits so-called ratiometric analysis of calcium. The term *ratio* in this context simply means that indo-1 fluoresces at 485 nm (turquoise) when free of calcium but at 405 nm (violet) in the chelated state. By using the ratio between violet and turquoise fluorescence, we can get a measure of the amount of calcium in a cell that is independent of the amount of dye loaded. Some flow cytometers can calculate the ratio between any two of their parameters. For others, this calculation has to be done through post-acquisition software.

Figure 11.4 shows a time course of this ratio (related to internal calcium) as it changes in response to the stimulation of lymphocytes by a ligand bound to their surface receptors. Such a time course can be performed on a flow cytometer by loading cells with indo-1 in advance and then stimulating them with a ligand (in a calcium-containing medium) immediately before placing the sample tube on the sample manifold. Alternatively, the sample manifold of many research cytometers can be modified to permit rapid injection of reagents into the sample tube while the sample is being drawn through the system. In either case, measured cell parameters can then be acquired over the subsequent minutes and their 405:485 fluorescence ratio determined.

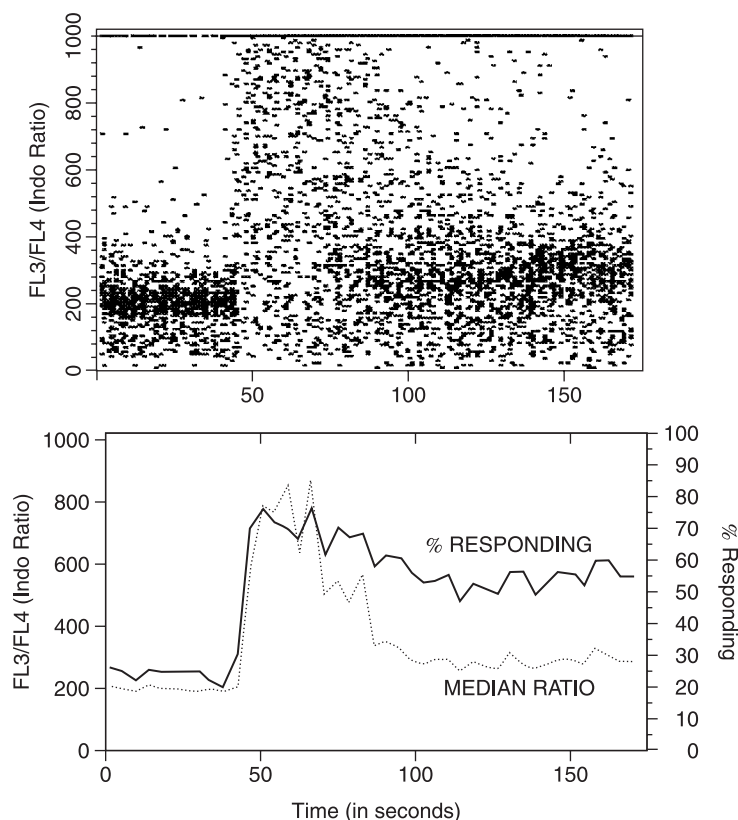


Fig. 11.4. The time course of a calcium response (measured by the indo-1 405/485 fluorescence ratio) induced in lymphocytes by the addition of a stimulus. The upper plot is a two-dimensional dot plot of the calcium ratio versus time. The lower plot shows data binned in 5 s intervals and processed to give both the median ratio and the percentage of cells above the base line as they change with time.

Of course, to talk about kinetic measurements, we need to bring in the parameter of time. Time is, in many ways, the hidden extra parameter of every flow system. Depending on the software being used, it will be more or less easy to access this time parameter for use in a kinetic profile. In the best case, each data file can have time as an extra parameter for each cell. We would be able to plot any other parameter(s) like turquoise and/or violet fluorescence against time

for each cell acquired into the computer memory, thus giving a time course of the change of that parameter during the course of the sample acquisition. It is also possible to use software to add a time parameter to a data file. Knowing, from the file header information, the start and finish time of the file, the software assumes constant cell flow rate and assigns a time to the passage of each cell through the laser beam.

This calcium ion procedure is an example of a class of protocols that measure the kinetics of functional activity in living cells. They depend on the ability to find a compound able to enter living cells and then alter its fluorescence in relation to a changing intracellular environment. As we have seen, compounds are available that can be used to assay calcium ion concentration. Other compounds will alter their fluorescent properties in response to changes in pH or to changes in membrane potential. Reduced fluorochromes such as dichlorofluorescein diacetate, which fluoresce only when oxidized, have been used to measure the production of peroxides during neutrophil activation. Almost everyone starts out in flow cytometry with work on static systems. A shift of direction into the realm of function and kinetic analysis requires a broadening of one's entrenched ideas of what flow cytometry is all about. The shift is almost certain to prove both challenging and informative.

THE AQUATIC ENVIRONMENT

Blood has been an ideal object for flow cytometric attention in large part because it occurs naturally as a mixed population of single cells. Lakes and oceans are also suspensions of mixed types of single cells. As such, they would appear to present an obvious target for flow analysis. Indeed, flow cytometers are now in place in many marine laboratories and on board sea-going vessels.

Aquatic single cell organisms with a size range of about 0.02–200 μm in diameter occur in nature in concentrations that range from about 10^2 to 10^7 per ml. They include viruses, bacteria, cyanobacteria (formerly known as *blue-green algae*), autotrophic phytoplankton (unicellular plants), and heterotrophic zooplankton (unicellular animals). The analysis of aquatic organisms by flow cytometry presents

some characteristic features that may serve to highlight the issues that, to a greater or lesser extent, affect all flow analyses. In the first place, because of the presence of naturally occurring photosynthetic pigments, the phytoplankton are highly autofluorescent (recall that some of them contain phycoerythrin, peridinin-chlorophyll complexes, or allophycocyanin). This autofluorescence leads to high background intensity against which positive staining of low intensity may be difficult to detect. The autofluorescence is also variable and may depend on the environment or metabolic state of the cell. The autofluorescence can, however, be exploited and used to distinguish different classes of organisms and different metabolic states.

Another characteristic of the aquatic environment is that the abundance of organisms of different types is highly variable; aquatic scientists do not have the benchmarks of a fairly tight “normal range” that clinical scientists depend on. In addition, the abundance of very small cells in the aquatic environment presents a challenge in instrument tuning and sensitivity. Not all cytometers can distinguish the forward scatter signal of nano- or picoplankton from optical noise or from particulate matter in the sheath stream. Because of the great size heterogeneity of plankton, a cytometer for aquatic analysis must be able to cope with both small and large particles at the same time. A final problem is that the most common particles in aquatic samples are not living; they represent decaying organic matter, silica- or calcium-containing empty cell walls, and suspended sediment, which are all difficult for a flow cytometer to distinguish from living cells.

Despite these problems, flow cytometry has had some noted success in aquatic research, particularly in relation to studies on the phytoplankton. Because all phytoplankton possess chlorophyll, but only the cyanobacteria possess the phycobiliproteins, autofluorescence “signatures” from water samples, based on the chlorophyll (fluorescence >630 nm), phycoerythrin (fluorescence <590 nm), and forward scatter of particles, have been used to characterize the changes that occur in plankton at different depths or at different locations (Figs. 11.5 and 11.6).

Figure 11.7 shows an example of the way in which flow cytometric analysis can distinguish six different species of plankton in culture and define which of these species are favored by grazing marine scallops as a source of food. Results such as these have been used to

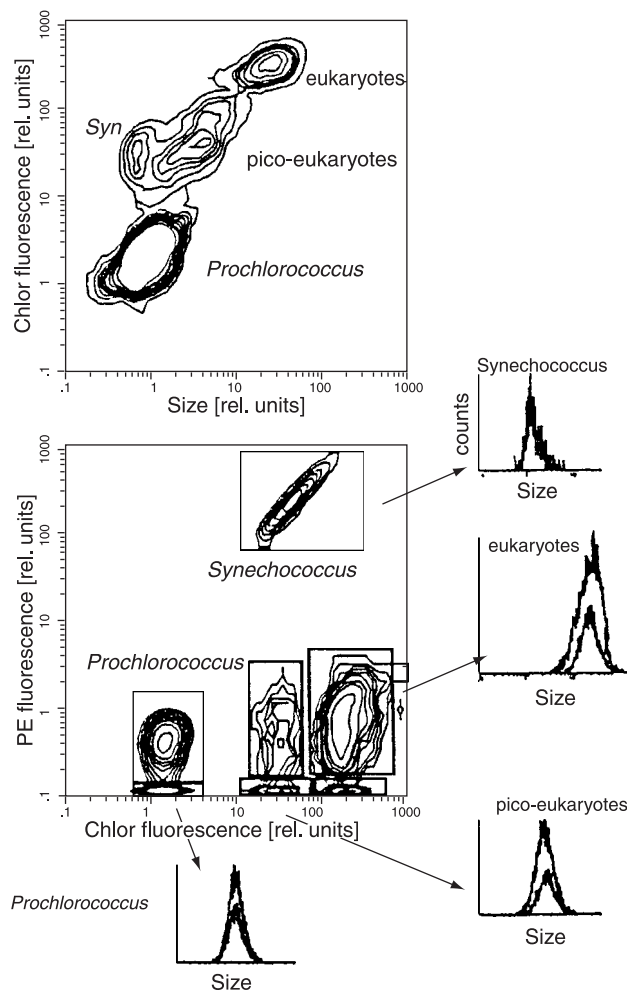


Fig. 11.5. The flow cytometric signature of a seawater sample taken at 90 m depth in the North Atlantic. Chlorophyll autofluorescence (>650 nm) has been plotted against side scatter ("size") and against phycoerythrin autofluorescence (530–590 nm). From Veldhuis and Kraay (2000).

suggest modifications in the menu supplied to scallops being farmed in aquaculture tanks.

Flow cytometry has also led to the notable discovery, reported by Sallie Chisholm in 1988, of the existence of a novel group of small,

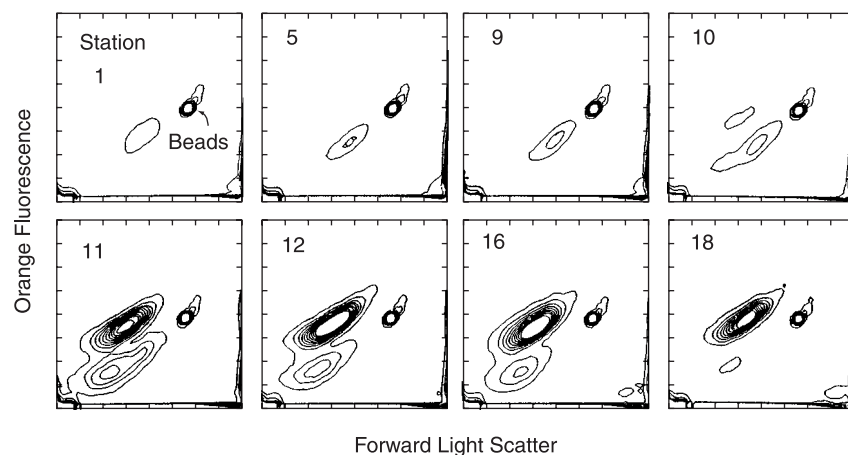


Fig. 11.6. Flow cytometric analysis of surface water from points at 1.5 mile intervals off shore from Cape Hatteras, North Carolina. Forward scatter and orange auto-fluorescence identify two *Synechococcus* populations with different phycoerythrin content. Beads were used to calibrate the number of cells present. From Chisholm et al. (1986).

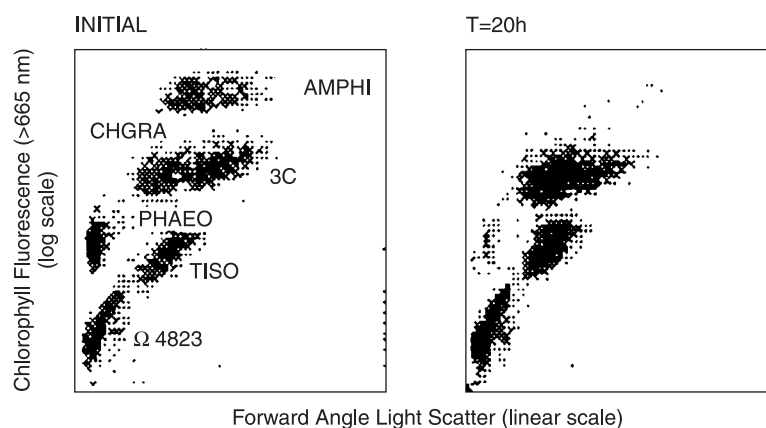


Fig. 11.7. Small scallops were placed in tanks with six species of phytoplankton that show distinctive flow cytometric signatures. After 20 h of grazing, it was apparent from the differences between the flow dot plots that the scallops had exhibited definite preference for two of the six species. Courtesy of Sandra Shumway.

prokaryotic phytoplankton; these free-living, marine prochlorophytes (*Prochlorococcus*) are between 0.6 and 0.8 μm in size but possess pigments more like those of eukaryotic plants than of other prokaryotes. The use of shipboard flow cytometry during cruises off southern California, the Panama Basin, the Gulf of Mexico, the Caribbean, and the North Atlantic between Woods Hole, Massachusetts and Dakar, Senegal (who said flow cytometry isn't fun?) has found these previously unknown prochlorophytes in remarkable abundance and indicated that they may be responsible for a significant portion of the global photosynthetic productivity of the deep ocean. More recently, Claude Courties (1994) has used flow cytometry on samples from the Thau lagoon in the Mediterranean off the coast of France to detect very small, eukaryotic picoplankton (about 1 μm) that have been called "the smallest eukaryotic organisms."

The CytoBuoy project from the European Community Marine Science and Technology Programme has been developing prototypes for a small flow cytometer (a 38×48 cm cylinder) that fits in ocean buoys. Current implementation of the CytoBuoy includes a cytometer inside a buoy, sampling just below the water surface. Forward scatter, side scatter, and orange fluorescence signals from particles are collected. It is projected that the CytoBuoy could take samples in the ocean at depths up to 500 m. Data transmission for continuous monitoring of ocean plankton could occur via short wave over a distance of up to 50 km. At a rate of about 20 bytes per second, this would give a sampling frequency of about one sample per hour.

The problems presented by the heterogeneity of the aquatic environment and the instrumental and conceptual developments made by aquatic scientists toward handling these problems have led to advances that can enrich the work done in all fields of flow analysis. Cytometers that can deal with the instability of the ocean environment will be all the more dependable in a relatively stationary land-based laboratory. Cytometers that are developed to handle both very small and very large particles may allow flow analysis to move more decisively into the fields of microbiology, parasitology, mycology, and botany. The general idea of studying autofluorescence instead of trying to avoid or ignore it is one that may be profitably considered by cytometrists in many areas of endeavor.

REPORTER MOLECULES

The Herzenbergs' group at Stanford has developed a flow cytometric method for assaying the presence of the enzyme β -galactosidase (coded by the *lacZ* gene from *Escherichia coli*). The presence of this enzyme can be detected by use of a so-called fluorogenic substrate—in this case fluorescein digalactopyranoside (FDG), which is cleaved by β -galactosidase to fluorescein monogalactoside and then to fluorescein, which is fluorescent. The importance of assaying for the presence of β -galactosidase transcends any interest in regulation of expression of this enzyme in bacterial cells: The *lacZ* gene has been used extensively in molecular biology as a reporter for the presence and/or expression of recombinant genes in eukaryotic cells. Cloned genes can be inserted, along with the *lacZ* bacterial gene, into eukaryotic cells; if they are all under the control of the same promoter, expression of the *lacZ* gene will then become a marker for the expression of the cloned genes.

A creative use of this technique was developed by Nolan and Krasnow at Stanford in a system they called *whole animal cell sorting* (WACS). The system does not involve sorting of intact animals (sheep to the left, goats to the right) but rather sorting of all the cells from a whole animal, after they have been dissociated. Specifically, the system has been used to study development of the fruit fly *Drosophila*. Identifiable cell types in developing *Drosophila* embryos have specific promoter regions in their genome that become activated in the course of development to initiate the formation of gene products typical of each cell type. Embryos can be transfected with *lacZ* into chromosome positions driven by a cell-type-specific promoter. These embryos (containing the introduced *lacZ* gene under the control of a specific promoter) are then grown to a given developmental stage. The cells expressing the reporter gene will contain β -galactosidase. Depending on the promoter gene governing the *lacZ* gene, different types of cells will therefore fluoresce when loaded with FDG. The distribution of these fluorescent cells (and therefore the activity of the specific promoter) can be visualized by looking at the intact embryo (Fig. 11.8). The embryos can also, however, be dissociated and the cells expressing the reporter gene then sorted by flow cytometry, based on fluorescein fluorescence intensity (Fig. 11.9). In this way,

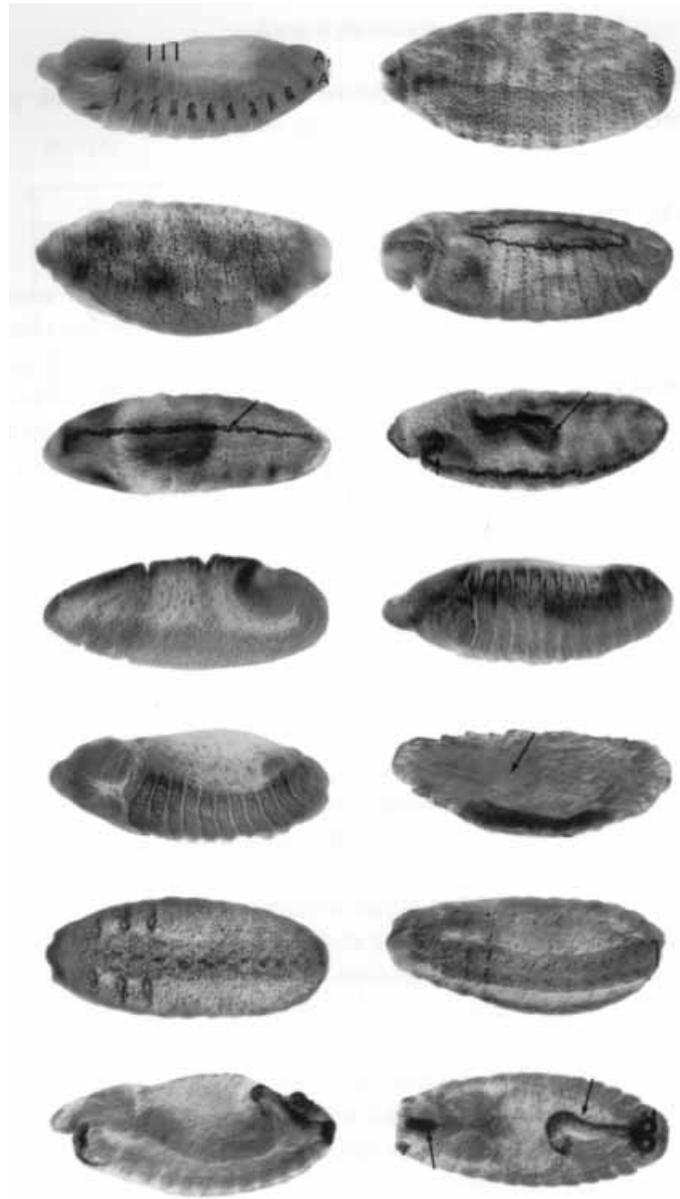


Fig. 11.8. *Drosophila* embryos transfected with the *lacZ* gene into association with different cell-type-specific promoters. Depending on the promoter gene, cells in different patterns over the embryo surface will possess the enzyme β -galactosidase (indicated by dark grains in this photograph). Courtesy of YN Jan from Bier et al. (1989).

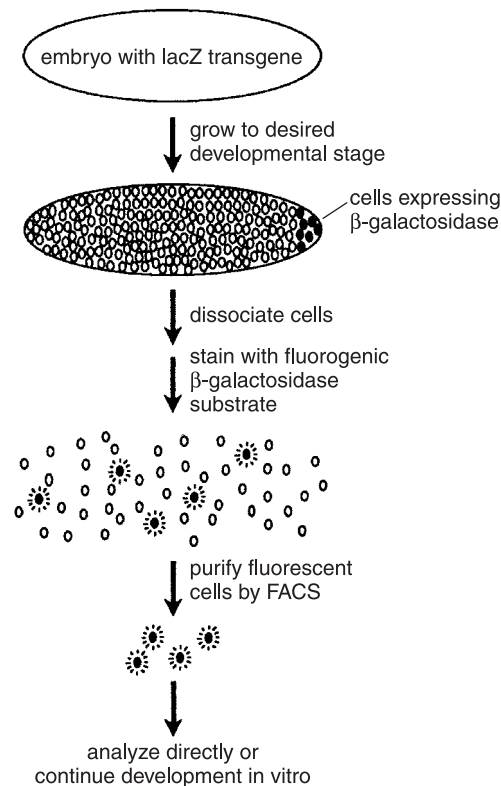


Fig. 11.9. The experimental protocol for whole animal cell sorting (WACS). Courtesy of Mark Krasnow.

cells destined for different functions can be purified and their subsequent development and interactions with other cells observed in culture (Fig. 11.10).

Over the past several years, the jellyfish *Aequorea victoria* has had remarkable impact on the field of reporter molecules in biology. When calcium ions bind to one of its proteins (aequorin), light is emitted. In vitro, aequorin emits blue light when binding calcium. The jellyfish, however, produces green light because a second protein (the imaginatively named “green fluorescent protein” or GFP) receives energy from aequorin and then emits fluorescence at a longer wavelength (remember those tandem fluorochromes). In a landmark paper by Chalfie et al. (1994), GFP was shown to remain fluorescent when transfected into the bacterium *Escherichia coli* (under the con-

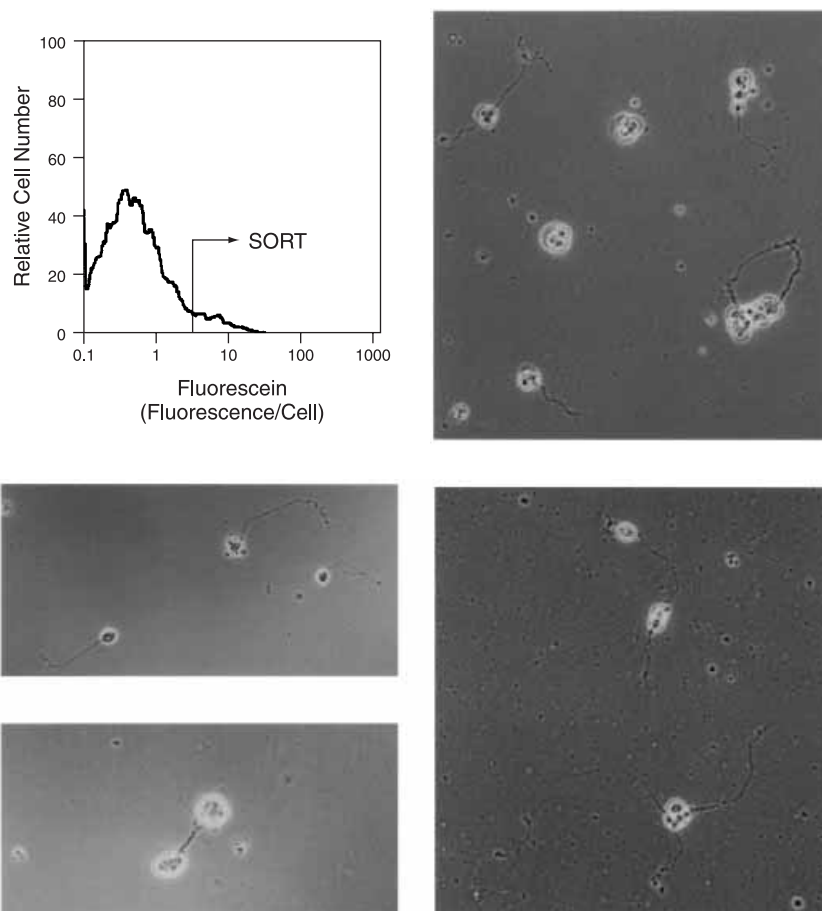


Fig. 11.10. When *lacZ* has been transfected into *Drosophila* embryos in association with a neuronal-cell-specific promoter, the cells that fluoresce brightly in the presence of fluorogenic β -galactosidase substrate will, when sorted, develop neuronal processes in culture. From Krasnow et al. (1991). *Science* 251:81–85. Copyright AAAS.

trol of an exogenous inducer) and into the nematode *Caenorhabditis elegans* (under the control of a cell-lineage-related promoter). Subsequent work in this field has elaborated a series of GFP-derived fluorochromes of different colors (BFP, CFP, and YFP). They have been used as reporters to detect induction of different proteins or, alternatively, have been engineered as fusion proteins so that, without

staining, the presence of these proteins can be assayed by flow cytometry and their cellular location visualized by microscopy. Flow sorting has had an important role to play in the development of stable GFP-fusion protein transfected cell lines (Fig. 9.4 shows an example of sorting for GFP fluorescence).

MICROBIOLOGY

Although microorganisms would seem to be ideal candidates for flow analysis, flow cytometry has been slow to make its presence felt in the field of microbiology. To a great extent, this is attributable to limitations of the instrumentation; flow chambers and sheath fluid and electronics designed for eukaryotic cells have often not worked dependably well with the smaller members of our universe. Without the help of autofluorescent pigments that aid oceanographers in the identification of phytoplankton, microbiologists have difficulty in resolving low-intensity scatter and fluorescence signals from debris and instrument noise. A considerable amount of work has aimed at overcoming some of these instrumental difficulties; many cytometers, if well-aligned, perform acceptably with small bacterial cells.

Much of the work on microorganisms in flow systems has concerned yeast, algae, and protozoa; although smaller than mammalian cells, these eukaryotes are considerably larger than most bacteria. DNA, RNA, protein, and light scatter measurements have been made on these organisms, and the feasibility of cell cycle analysis has been demonstrated. Bacteria, however, present more acute difficulties. The diameter of bacterial cells is perhaps 1 μm (compared with 10 μm for mammalian blood cells), and therefore the surface area to be stained (and resulting fluorescence intensity) is 10^2 less than that of a mammalian cell. The DNA content of the *E. coli* genome is about 10^{-3} times that of a diploid human cell. Hence, bright dyes and sensitive instrumentation are required for studies of bacteria. Nevertheless, reasonable DNA histograms of bacteria can be obtained by flow cytometry. Methods are being developed to investigate cell cycle kinetics, the effects of antibiotics, and the detection and identification of bacteria for clinical investigations.

However, a technique developed at the Massachusetts Institute of

Technology has approached the problem with the “if you can’t beat them, join them” philosophy of allowing bacteria to masquerade as larger particles. The technique involves the creation of “salad oil” emulsions of drops of agar within bacterial suspensions in buffer solution. By adjusting the size of the drops and the concentration of the bacteria, it is possible to arrange conditions so that, on average, each drop of gel contains one bacterial cell. The microdrop then becomes a minicontainer for the bacterial cell, allowing the diffusion of stain, nutrient, antibiotics, and so forth, but containing the bacterial cell and all its progeny.

The usefulness of this technique has been shown by its ability to detect the division of these bacterial cells. By staining the cells within the droplets in some way (e.g., for DNA or protein content), the original culture will form a single flow histogram peak representing gel microdroplets, each fluorescing with an intensity related to the DNA or protein content of its entrapped single bacterial cell. After one replication cycle in which all the bacteria are replicating, each droplet will then contain two cells and have twice the original fluorescence intensity. Alternatively, if only some of the bacteria are replicating, a small population of gel droplets with twice the fluorescence intensity will appear. The droplets containing replicating cells will then progress to 4-fold, 8-fold, and 16-fold intensity as replication continues (Fig. 11.11). The technique can provide a sensitive method for studying small particles as well as a very rapid assay for the replication of a small proportion of bacterial cells in the presence of antibiotics, growth factors, or varied growth conditions (Fig. 11.12).

Although this gel microdroplet method is still new (even after 10 years) and its potential applications relatively untested, it has been described here because it can teach us certain general lessons. It serves to remind us that cytometry, despite its name, does not necessarily involve the flow analysis of cells; particles of many sorts will do just as well. It is also of interest as a method that has, in fact, institutionalized the formation of clumped cells that most workers try so hard to avoid. In addition, it has provided us with a way to make a small cell into a larger (flow-friendly) particle. Finally, it has given us inspiration by exemplifying the way in which lateral thinking can extend the impact of flow cytometry in new directions.

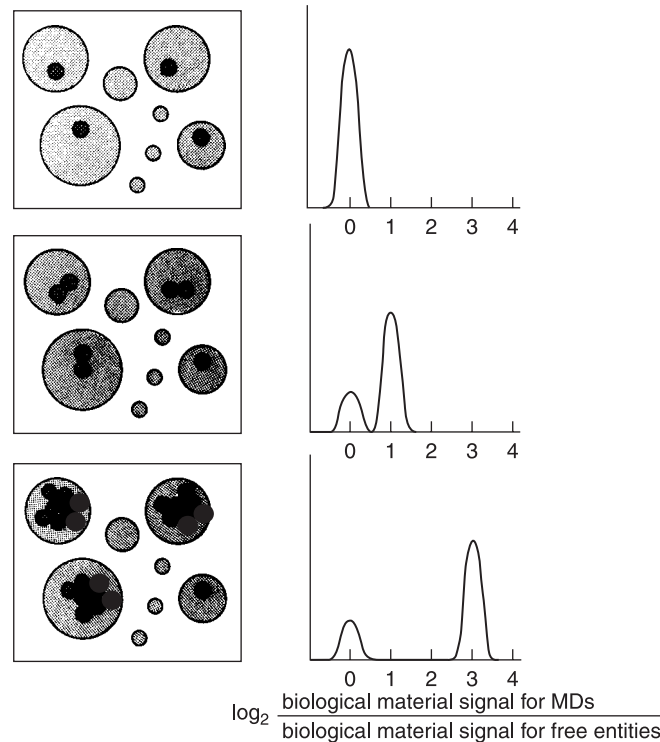


Fig. 11.11. Illustration of the use of gel microdroplets for sensing growth at the level of one cell growing into a two-cell microcolony. By staining the cells within microdroplets with, for example, a DNA-specific fluorochrome, a small subpopulation of cells dividing more or less rapidly than most could be detected in a flow histogram of microdroplet fluorescence. From Weaver (1990).

MOLECULAR BIOLOGY

Flow cytometry has been applied in many creative ways to the science of molecular biology. Flow sorting, based on Hoechst 33258 and chromomycin A3 fluorescence, turns out to be one of the best ways available for obtaining relatively pure preparations of each type of chromosome. Even those chromosomes that are not distinguishable by their fluorescence (e.g., 9-12) can usually be sorted from hamster-human hybrid cell lines. These preparations of reasonably

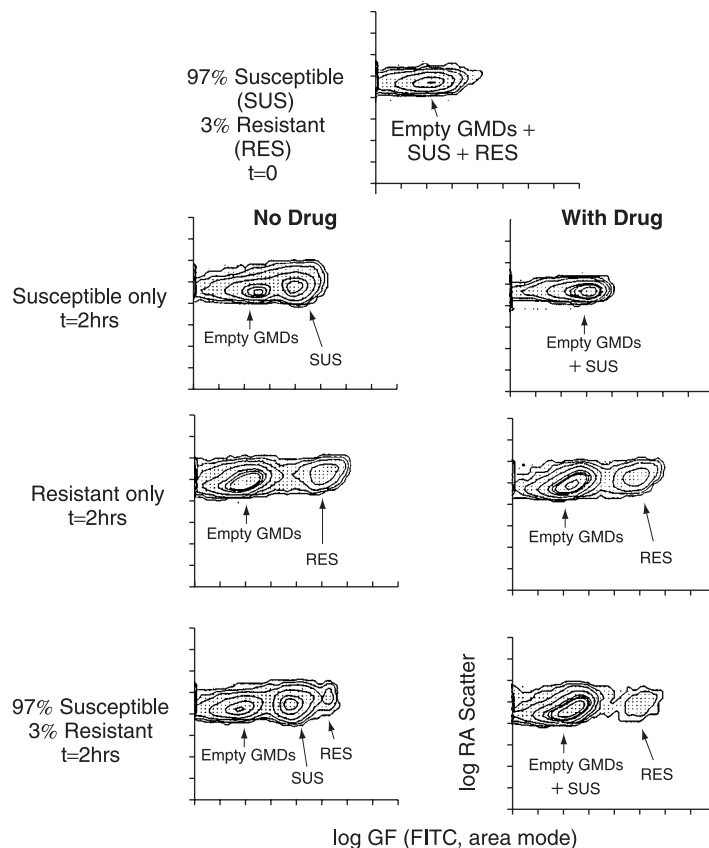


Fig. 11.12. The use of gel microdroplets and flow cytometry to assay drug sensitivity of bacterial cells. The figure shows side scatter and green fluorescence contour plots of gel microdroplets (GMDs) containing *E. coli* cells that have been stained with fluorescein isothiocyanate for total protein. The microdroplets have been analyzed in the flow cytometer either at time 0 or 2 h after incubation in control medium (left plots) or medium containing penicillin (right plots). A model system was created by mixing two strains of bacteria (susceptible or resistant to penicillin). The data show that a small subpopulation of resistant cells could be detected within 2 h because of its rapid growth in comparison to susceptible cells. From Weaver et al. (1991).

pure flow-sorted chromosomes have been the starting material for obtaining chromosome-specific DNA libraries. The Human Genome Project has made extensive use of chromosomes obtained with the sorting cytometers at Los Alamos and Livermore. The bottleneck in

TABLE 11.1. Chromosome Sorting

Application	Chromosomes required	
Polymerase chain reaction chromosome paints	3.0×10^2	
Viral cloning (17 kb inserts)	1.6×10^6	
Cosmid cloning (37 kb inserts)	6.4×10^6	
Yeast artificial chromosome cloning	6.4×10^7	
Sorter	Chromosomes sorted per 24 h day	Estimated sorting time for YAC cloning (6.4×10^7 chromosomes)
Conventional	3×10^6	21 days
High speed	1.5×10^7	4 days
Optical zapper	7.5×10^7	0.8 days

After Roslaniec MC et al. (1997). Hum. Cell 10: 3–10.

this technique is the time it takes for sorting. In a conventional sorter, if the flow rate is limited to about 1000 particles per second, then human chromosomes of a particular type could theoretically be sorted at a rate of about 40 per second ($1000/23$). Thus it would take about 7 h to obtain 10^6 chromosomes of a given type under optimal conditions. The high-speed drop sorters at Los Alamos and Livermore were developed with the demands of the Human Genome Project in mind. The technique for optical (zapper inactivation) sorting is the next step in this evolution (Table 11.1).

Even small numbers (20,000 or so) of sorted chromosomes can be used quite neatly to aid in the mapping of genes to chromosomes. Chromosomes of each type are simply sorted (two at a time: one type to the left, the other to the right) onto a nitrocellulose filter. The DNA is then denatured on the filter where it can be hybridized to a radioactive gene probe. Autoradiography of the filter will then reveal whether the probe has hybridized to the DNA from any given chromosome (Fig. 11.13). In this way, the sorting of small numbers of each of the chromosomes onto filters allows the mapping of any available gene probe to its chromosome.

Much like chromosomes, DNA fragments generated by restriction enzyme digestion of native DNA, when stained with DNA-specific

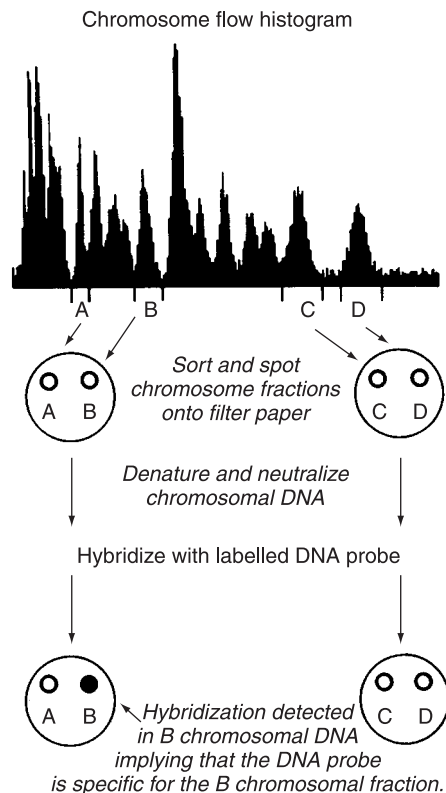


Fig. 11.13. DNA blot analysis using spots from chromosomes sorted directly onto filter paper on the basis of their Hoechst 33258 fluorescence. From Van Dilla et al. (1990).

fluorochromes, fluoresce more or less brightly according to their length. Flow data acquisition could, in theory, provide histograms indicating the relative proportions of different-sized fragments in a digest. Implementation of this application has not been trivial because, compared with chromosomes, fragments of DNA are much smaller and fluoresce much less brightly. Rising to this challenge, scientists have developed ultrasensitive flow cytometric systems by using high-efficiency optics and light detection, bright fluorochromes, and very slow sheath velocities of approximately 2–4 cm per second (compare this with the usual cytometers with velocities of 10 m [1000 cm] per second). With slow flow rates, DNA fragments spend longer

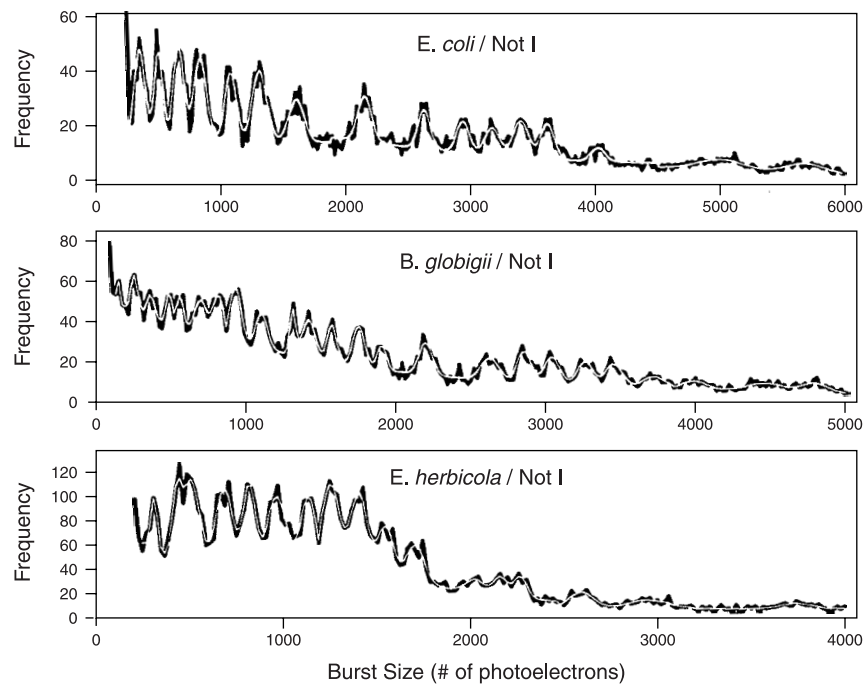


Fig. 11.14. Flow cytometric signatures distinguishing three different strains of bacteria according to the size distributions of DNA fragments generated by restriction enzyme digestion. From Kim et al. (1999).

time in the laser beam, thereby emitting more fluorescence per fragment and increasing instrument sensitivity for detection of short fragments with low intensities. As an alternative to pulsed-field gel electrophoresis, the flow methodology records similar information about the distribution of fragment sizes, but with better resolution, greater accuracy, and higher sensitivity. In addition the flow method requires less DNA and less time. In one proof-of-principle application of this technology, when DNA from bacteria has been broken into fragments by digestion with restriction enzymes, the size distribution of the fragments is typical of a given strain of bacteria. Therefore the flow histogram that is derived from sending the fragments through a flow cytometer is a signature of the particular bacterial strain and can be used for identification (Fig. 11.14).

Workers with James Jett at Los Alamos have been developing cytometers that are able to detect the fluorescence from single molecules and have been applying this capability to the problem of DNA sequencing. The proposed technique involves the synthesis of a complementary strand of DNA with fluorescently tagged precursors (each base of a different color). The labeled (fluorescent) duplex DNA molecule is then attached to a microsphere and the microsphere suspended in a flow stream where it can be sequentially cleaved with an exonuclease. The cleaved fluorescent bases from the molecule would then be identified by their color as they pass through the laser beam, and the sequence in which the colors appear will reflect the base sequence in the DNA. It is projected that between 100 and 1000 bases per second could be sequenced, although there are still obstacles to be surmounted before this technique becomes a practical reality.

MULTIPLEX CYTOMETRY FOR SOLUBLE ANALYTES

With a continuation of the lateral thinking that has marked developments in flow cytometry for many years, it is now possible to use a simple flow cytometer to analyze multiple soluble analytes in a single test tube. Sets of beads can be manufactured with distinctive ratios of two different fluorochromes (for example, red and orange). By precisely controlling the ratios of these two dyes, bead sets can be obtained with 100 different specifications (according to their red/orange balance). When run on a flow cytometer, the beads cluster into 100 separate regions on a two-color (red vs. orange) dot plot (Fig. 11.15). In a multiplexed version of an ELISA assay, each bead type can be linked to a different capture molecule; the capture molecule will bind a soluble target analyte to the bead (compare this with the solid-phase capture molecules on an ELISA plate). For example, capture molecules on the beads might be a range of allergens (to bind antibodies from the serum for allergy profiling) or nucleotide probes for single-nucleotide polymorphisms (SNPs) or antibodies to a range of cytokines.

After preparation (with beads of each red/orange ratio having been linked to capture molecules for specific targets), the beads can all be combined in a single tube. For the assay, a test solution is added to the mixed bead suspension. After incubation, centrifugation,

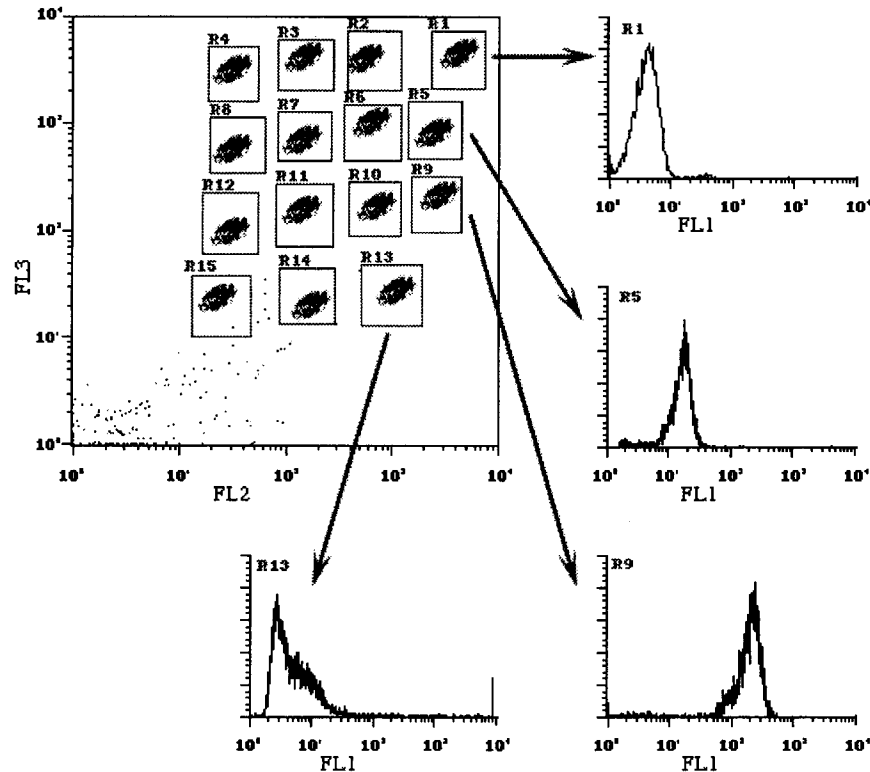


Fig. 11.15. A multiplex bead array that can be used to capture multiple soluble analytes. In the dot plot, 15 types of beads are distinguished by their red/orange ratios; they capture 15 different cytokines. For example, the beads in Region 1 capture interleukin (IL)-9; in Region 5, IL-2; in Region 9, IL-5; and in Region 13, MCP-1. The green fluorescence histograms from each region show, by their intensity, how much cytokine has been captured by each type of bead. Standard curves can relate the green fluorescence intensity to the concentration of cytokine in the sample. The experimental format illustrated in the cartoon here is patterned directly from work by RT Carson and DAA Vignali (1999).

and washing, fluorescein-conjugated detection reagents are added to the incubation mixture. Much like the top layer in an ELISA assay, the detection reagents will bind to the target analytes on the beads. After running the tube of beads through the flow cytometer, the final read out comes from gating on each one of the 100 types of beads in turn and then (knowing the particular capture antibody on that type of bead) reading the green fluorescence intensity on that red/orange

bead cluster. The intensity of the green fluorescence will, under careful conditions, correlate with the concentration in solution of the particular analyte that has been captured.

With this methodology, it is possible to assay for a large number of cytokines simultaneously in one tube or to test for a complete selection of clinically relevant enzymes or for antibodies to a series of allergens. In addition, by using one color bead type for each patient and then mixing the samples together for analysis, many patients can be assayed for a particular analyte in a single test tube. Each red/orange cluster will mark the results from a particular patient.

REPRODUCTIVE TECHNOLOGY

Prenatal Diagnosis

Prenatal diagnosis of fetal abnormalities conventionally involves testing of cells from either the amniotic fluid or the chorionic villus. Both techniques involve sampling of tissues closely associated with the fetus; they therefore pose some risk of causing miscarriage of the pregnancy. For this reason, sampling fetal cells noninvasively has been a long-held goal.

Small numbers of fetal erythrocytes that have escaped through the placenta exist in the maternal circulation. These cells can be detected by their fetal hemoglobin (which distinguishes them from most maternal erythrocytes). Their presence in significant numbers (greater than 0.6%) has been used to diagnose a fetal–maternal hemorrhage across the placenta and to mandate clinical intervention to prevent the development in the Rh-negative mother of anti-Rh antibodies (see Chapter 10). Because fetal cells are accessible by drawing blood from the mother, sampling of them from the mother's circulation involves no hazard to the fetus and holds the possibility of noninvasive prenatal diagnosis of fetal abnormalities. However, prenatal diagnosis requires DNA, and most of the fetal erythrocytes in the maternal circulation are not nucleated. Over the past decade, work in this field (led, to a great extent, by Diana Bianchi at Tufts University in Boston, Massachusetts) has centered on the development of methods for distinguishing nucleated fetal cells from maternal cells and from non-nucleated fetal cells and then on methods for isolating these cells

for subsequent fluorescence in situ hybridization (FISH) analysis of the fetal genome for disease-related sequences. Because the nucleated fetal cells are very rare (estimated to be fewer than 20 nucleated fetal erythrocytes per 20 ml of maternal peripheral blood; that is, a cell ratio of about $1:10^9$), flow sorting has been proposed as a method for purifying or at least enriching the population before FISH analysis.

Such sorting requires a flow-detectable method for classifying nucleated fetal cells. Two protein markers, among many others, that have been proposed to distinguish fetal cells from maternal cells are the transferrin receptor and fetal hemoglobin. Antibodies against fetal hemoglobin have been used (by Bianchi) in conjunction with a Hoechst stain for DNA to detect only those cells that have fetal hemoglobin and are also nucleated. As a model system for the reliability of flow sorting to enrich for fetal cells, blood from women carrying male fetuses has been studied. The presumptive fetal cells from the maternal blood are sorted (based on Hoechst and anti-fetal hemoglobin staining) and then tested with chromosome-specific FISH probes for the presence of a Y chromosome or for only a single X-chromosome (proving that the sorted cells were, indeed, from the male fetus and not from the mother). Results from these experiments are promising. Although these techniques are still at early stages of investigation, there is considerable optimism that flow sorting of fetal cells may fulfill the need for prenatal diagnosis of fetal abnormalities without the ethical problems posed by invasive sampling of low risk pregnancies.

Determining Sex

In another facet of reproductive technology, flow sorting has been used to separate sperm bearing X and Y chromosomes. If you own a dairy herd, you want your cows to produce more cows (and few bulls). Conversely, beef farmers want males. Because bovine X chromosomes are considerably larger than Y chromosomes, sperm bearing X chromosomes have approximately 4% more DNA than their Y bearing brothers. By staining with a Hoechst dye, the two classes of sperm can be sorted, with a flow cytometer, according to their fluorescence intensity. Sex determination of the offspring, resulting from insemination with the sorted sperm, is not perfect (think of the pos-

sibility of overlap between the intensities of sperm that are separated by only 4%), but it is not bad. There is now a commercial flow cytometer that has been developed specifically for sorting animal sperm. Sex-selected calves are being produced more or less routinely by artificial insemination with sorted sperm. The technology has also been applied to the high-stakes world of race horse breeding.

Speaking of high-stakes worlds, there is now at least one commercial clinic that provides sperm sorting for humans (“for prevention of genetic disorders” or “to increase their probability of having a child of the less represented sex in the family”). Although human sperm bearing X and Y chromosomes differ in DNA content (and fluorescence intensity) by only 2.8%, the technique does appear to work with some reliability. In a 1998 summary from the clinic, 14 pregnancies from insemination with X-bearing sperm were reported: 92.9% of those were female as desired. The procedure is not inexpensive, it may have arguable ethical implications, and it is not perfect. The question remains as to whether it could be a glimpse of the future. If it is, then we need to ponder the fact that most American families seem to want daughters. . . .

FURTHER READING

Section 9 in **Current Protocols in Cytometry** provides methods and some theory on many flow cytometric functional assays (including those mentioned here and many others). Chapter 20 in **Darzynkiewicz** is a discussion of methods related to staining lymphocytes for activation antigens.

A special issue of *Cytometry* (Vol. 10, No. 5, 1989) is devoted to “Cytometry in Aquatic Sciences.” A more recent issue of *Scientia Marina* (Vol. 64, No. 2, 2000) is also devoted to the same subject: “Aquatic Flow Cytometry: Achievements and Prospects.” In addition, Chapter 31 of **Melamed et al.** discusses the application of flow cytometry to higher plant systems.

Section 4 in **Diamond and DeMaggio** has several methodological chapters discussing reporter genes, GFP, and cell tracking dyes. The classic reference on GFP is by Chalfie M, et al. (1994). Green fluorescent protein as a marker for gene expression. *Science* 263:802–805. A good review of the uses of flow cytometry for GFP analysis is by Galbraith DW, Anderson MT, Herzenberg LA (1999). Flow cytometric analysis and FACS sorting of cells based on GFP accumulation. *Methods Cell Biol.* 58:315–339.

Discussion of the WACS methodology can be found in Krasnow MA, Cumberledge S, Manning G, et al. (1991). Whole animal cell sorting of *Drosophila* embryos. *Science* 251:81–85.

The gel microdroplet technique is described in articles by Powell KT, Weaver JC (1990). Gel microdroplets and flow cytometry: Rapid determination of antibody secretion by individual cells within a cell population. *Bio/Technology* 8:333–337; and Weaver JC, Bliss JG, Powell KT, et al. (1991). Rapid clonal growth measurements at the single cell level. *Bio/Technology* 9:873.

Research on flow cytometry of microorganisms is reviewed in Chapter 29 of **Melamed et al.** and in Section XI in **Darzynkiewicz**. Microbiological methods are described in Section 11 of **Current Protocols in Cytometry**. A useful book edited by D Lloyd (1993) is *Flow Cytometry in Microbiology* (Springer-Verlag, London).

A good reference on the flow cytometry of DNA fragments is Kim Y, Jett JH, Larson EJ, et al. (1999). Bacterial fingerprinting by flow cytometry: Bacterial species discrimination. *Cytometry* 36:324–332. The proposed technique for sequencing DNA is described in a paper by Jett JH, Keller RA, Martin JC, et al. (1989). High-speed DNA sequencing: An approach based upon fluorescence detection of single molecules. *J. Biomol. Struct. Dynamics* 7:301–309.

Results using multiplexed bead flow cytometry have been reported by Carson RT, Vignali DAA (1999). Simultaneous quantitation of 15 cytokines using a multiplexed flow cytometric assay. *J. Immunol. Methods* 227:41–52.

A review of progress in developing flow methods for prenatal diagnosis is by Bianchi DW (1999). Fetal cells in the maternal circulation: Feasibility for prenatal diagnosis (review). *Br. J. Haematol.* 105:574–583. In addition, Chapter 20 by JF Leary in **Darzynkiewicz, 1994** discusses general issues of rare cell cytometry as well as specific reference to the sorting of fetal cells.

A provocative, nontechnical article on the ability to choose, with the aid of a flow cytometer, the sex of our children was written by L Belkin (“Getting the Girl” in *The New York Times Magazine*, July 25, 1999, pp 26–55). Results from a clinic that sells this technique to prospective parents have been reported by Fugger EF, Black SH, Keyvanfar K, Schulman JD (1998). Births of normal daughters after MicroSort sperm separation and intrauterine insemination, in-vitro fertilization, or intracytoplasmic sperm injection. *Hum. Reprod.* 13:2367–2370.